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Stat5a and Stat5b Proteins Have Essential and Nonessential, or Redundant, Roles in Cytokine Responses

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Summary

A variety of cytokines mediate the activation of *Janus* protein tyrosine kinases (Jaks). The Jaks then phosphorylate cellular substrates, including members of the signal transducers and activators of transcription (Stat) family of transcription factors. Among the Stats, the two highly related proteins, Stat5a and Stat5b, are activated by a variety of cytokines. To assess the role of the Stat5 proteins, mutant mice were derived that have the genes deleted individually or together. The phenotypes of the mice demonstrate an essential, and often redundant, role for the two Stat5 proteins in a spectrum of physiological responses associated with growth hormone and prolactin. Conversely, the responses to a variety of cytokines that activate the Stat5 proteins, including erythropoietin, are largely unaffected.

Introduction

Cytokines regulate the proliferation and/or differentiated functions of cells through their interaction with receptors of the cytokine receptor superfamily. These receptors mediate their diverse effects through their ability to associate with and activate members of the *Janus* family of protein tyrosine kinases (Jaks) (Darnell, Jr. et al., 1994; Ihle, 1995a, 1995b). Among the substrates of the Jaks are members of the signal transducers and activators of transcription (Stat) family of proteins (Ihle, 1996; Darnell, 1997). The Stats are recruited to the receptor complexes through SH2 domain recognition of specific sites of receptor tyrosine phosphorylation, are phosphorylated by the Jaks, undergo dimerization, translocate to the nucleus, and affect the expression of a wide range of genes.

Seven mammalian Stat family members have been identified, many of which play highly specific roles in innate and acquired immunity. Stat1 is critical for interferon (IFN)–induced viral resistance (Durbin et al., 1996; Meraz et al., 1996). Similarly, Stat6 specifically mediates the effects of IL-4 or IL-13 on B or T cells (Kaplan et al., 1996a; Shimoda et al., 1996), while Stat4 is critical for IL-12 signaling (Kaplan et al., 1996b; Thierfelder et al., 1996). The one possible exception is Stat3, which, when deleted, results in a very early embryonic lethality due to unknown deficiencies (Takeda et al., 1997).

Stat5 was identified as a prolactin-induced mammary gland transcription factor (Wakao et al., 1994). It was shown that two *Stat5* genes encode proteins that are approximately 95% identical in amino acid sequence (Liu et al., 1995) and that these two genes colocalized to murine chromosome 11, tightly linked to *Stat3* (Copeland et al., 1995). The identity and chromosomal colocalization suggest that the two genes resulted from a recent gene duplication. Since both genes are expressed in a variety of cell types, it was anticipated that they would be functionally redundant.

Stat5a and Stat5b proteins are activated by tyrosine phosphorylation in response to a wide variety of cytokines. In addition to prolactin, Stat5 proteins are activated by growth hormone (GH) (Gouilleux et al., 1995; Wood et al., 1995), erythropoietin (Epo) (Quelle et al., 1996), thrombopoietin (Tpo) (Pallard et al., 1995), interleukin 3 (IL-3) and GM-CSF (Mui et al., 1996), and interleukin 2 (IL-2) (Hou et al., 1995; Wakao et al., 1995). In addition, the activation of the Stat5 proteins has been implicated in bcr/abl transformation (Ilaria and Van Etten, 1996; Shuai et al., 1996) and transformation of lymphocytes to factor independence (Migone et al., 1995).

The role of Stat5 activation in erythroid proliferation or differentiation has been examined by various approaches. Receptor mutants have been used that are unable to recruit the Stat5 proteins to the receptor complex and thus fail to activate them. Our results (Quelle et al., 1996) indicated that Stat5 activation was not required for Epo-induced proliferation, while other studies (Damen et al., 1995; Gobert et al., 1996) concluded that Stat5 activation was required. The effects of dominant-negative forms of Stat5 have also been examined and used to implicate the Stat5 proteins in erythroid differentiation (Chretien et al., 1996; Iwatsuki et al., 1997; Wakao et al., 1997) as well as a role in IL-3–induced proliferation (Mui et al., 1996). However, in both cases, the effects were partial and, consequently, not definitive in establishing a critical, nonredundant role. Similar types of ambiguities exist in establishing a role for the Stat5 proteins in responses to other cytokines.

To assess the role of the Stat5 proteins, we derived mice in which the two genes are individually and simultaneously mutated. Because of the identity of the two proteins, it was assumed they would be redundant in function. Indeed, it was quite unexpected to find phenotypes associated with the disruption of the individual genes as recently reported for *Stat5a* (Liu et al., 1997) and, independently, for *Stat5b* (Udy et al., 1997). Our results support these findings but also demonstrate the loss of additional functions associated with growth hormone or prolactin. In addition, rather remarkably, the

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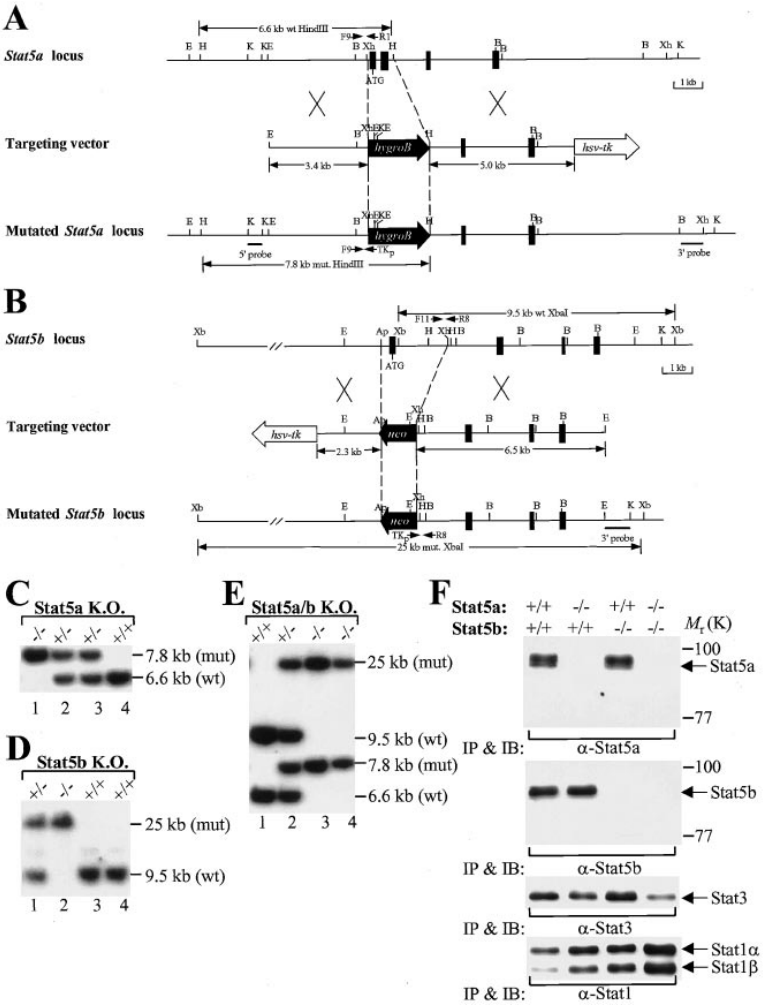

Figure 1. Generation of Stat5a$^{-/-}$, Stat5b$^{-/-}$, and Stat5a$^{-/-}$5b$^{-/-}$ Mice

(A) Structure of the 5′-end of the Stat5a locus, Stat5a-hygro targeting vector, and predicted Stat5a mutated locus.

(B) Structure of the 5′-end of the Stat5b locus, Stat5b-neo targeting vector, and predicted Stat5b mutated locus. Filled boxes indicate exons. The locations of the 5′ and 3′ external probes and PCR primers for genotyping are indicated. ATG denotes the first coding exon. Restriction enzyme sites shown are as follows: E, EcoRI; H, HindIII; K, KpnI; B, BamHI; Xh, XhoI; Xb, XbaI; Ap, ApaI.

(C) Southern blot analysis of HindIII-digested tail DNA Stat5a heterozygous F1 mice probed with a 5′ Kpn probe. The positions of the mutant 7.8 kb and wild-type 6.6 kb HindIII fragments are indicated. Mice homozygous (lane 1) and heterozygous (lanes 2 and 3) for the Stat5a mutation, and a wild-type mouse (lane 4) are shown.

(D) Southern blot analysis of XbaI-digested tail DNA from Stat5b heterozygous F1 mice probed with a 3′ EcoRI/KpnI probe. The positions of the mutant 25 kb and wild-type 9.5 kb XbaI fragments are indicated. Mice homozygous (lane 2) and heterozygous (lane 1) for the Stat5b mutation and wild-type mice (lanes 3 and 4) are shown.

(E) Southern blot analysis of XbaI-digested (upper panel) and HindIII-digested (lower panel) tail DNA from Stat5a/b heterozygous F1 mice. Size of fragments and probes used are the same as in (C) and (D). Mice doubly homozygous (lanes 3 and 4) and doubly heterozygous (lane 2) for the Stat5a/b mutation and a wild-type mouse (lane 1) are shown.

(F) Immunoprecipitation (IP) and Western blot analysis of Stat5a, Stat5b, Stat3, and Stat1 protein expression in liver extracts from Stat5a$^{+/+}$5b$^{+/+}$, Stat5a$^{-/-}$5b$^{+/+}$, Stat5a$^{+/+}$5b$^{-/-}$, and Stat5a$^{-/-}$5b$^{-/-}$ mice. Specific antisera used for the IP and immunoblot (IB) of the Stat proteins are indicated.

disruption of both genes failed to reveal a critical, nonredundant role in the response of a number of cytokines that activate the Stat5 proteins.

Results

Derivation and Initial Characterization of Mutant Mice

The chromosomal colocalization of the two *Stat5* genes necessitated the disruption of both alleles in ES cells to obtain mice that lacked both proteins. For comparison, mice lacking *Stat5a* or *Stat5b* alone were also generated. The strategies used are illustrated in Figures 1A and 1B. In both cases, targeting vectors were developed that delete the first coding exon and would generate a protein null phenotype. A hygromycin B–containing construct was used to target the *Stat5a* gene, while a neomycin containing construct was used to disrupt the *Stat5b* locus. To generate the Stat5a$^{+/-}$5b$^{+/-}$ ES cells, Stat5a$^{+/-}$ hygromycin-resistant ES cells were targeted in a second round with the Stat5b neomycin construct. Karyotypically normal, appropriately targeted ES cells were injected into blastocysts of C57Bl/6 mice. Germline transmission was obtained for two Stat5a, three Stat5b, and four Stat5a/b clones. Of the Stat5a/b germline animals, three were derived from clones in which targeting had occurred on the same chromosome. No differences in the phenotypes described below were observed among the independently derived mutant strains of mice.

Homozygously deleted animals were observed in the offspring of all mutant strains (Figures 1C, 1D, and 1E) at frequencies expected for normal Mendalian segregation (data not shown). However, approximately one-third of the Stat5a/b mutant mice die within 48 hr (data not shown). A number of possible reasons for mortality were examined, including altered blood glucose, triglycercides, cholesterol, urea nitrogen, and lactate, without identifying the basis for the mortality. In addition, there was not an apparent neurological basis for the mortality, since all the pups suckled and otherwise behaved normally.

The mutant strains were subsequently examined for

Table 1. In Vitro Colony Formation Ability of Bone Marrow Hematopoietic Progenitors from Stat5 Wild-Type and Mutant Mice in Response to Different Cytokines

		Genotype			
	Colony Type	Stat5a$^{+/+}$5b$^{+/+}$	Stat5a$^{-/-}$5b$^{+/+}$	Stat5a$^{+/+}$5b$^{-/-}$	Stat5a$^{-/-}$5b$^{-/-}$
Cytokine					
Epo	CFU-E	493 ± 110 (n = 16)	488 ± 96 (n = 4)	571 ± 84 (n = 6)	434 ± 76 (n = 7)
IL-3 + Epo	BFU-E	13 ± 6 (n = 16)	21 ± 3 (n = 4)	21 ± 5 (n = 6)	26 ± 8 (n = 7)
IL-3	CFU-Mix	319 ± 65 (n = 5)	302 ± 8 (n = 4)	310 ± 24 (n = 4)	182 ± 63 (n = 6)[a]
IL-5	CFU-Eos	12 ± 4 (n = 13)	10 ± 3 (n = 4)	18 ± 4 (n = 4)	3 ± 2 (n = 6)[a]
IL-7	Pre-B	41 ± 2 (n = 5)	38 ± 3 (n = 4)	38 ± 2 (n = 4)	11 ± 5 (n = 6)
Tpo	CFU-Mk	8 ± 5 (n = 11)	9 ± 2 (n = 5)	4 ± 2 (n = 4)	4 ± 2 (n = 6)
G-CSF	CFU-G	67 ± 20 (n = 14)	72 ± 28 (n = 4)	45 ± 25 (n = 5)	30 ± 23 (n = 6)
GM-CSF	CFU-GM	378 ± 91 (n = 11)	422 ± 68 (n = 6)	555 ± 113 (n = 4)	176 ± 42 (n = 6)[a]
CSF-1	CFU-M	605 ± 151 (n = 4)	655 ± 47 (n = 4)	608 ± 43 (n = 4)	691 ± 174 (n = 4)
SCF	CFU-Mix	48 ± 8 (n = 4)	42 ± 2 (n = 4)	41 ± 4 (n = 4)	35 ± 9 (n = 4)

Mean ± standard deviation of number of colonies/10^5 nucleated bone marrow cells.
[a] Size of colonies reduced compared to wild-type.

the presence of Stat5 proteins by immunoprecipitation and Western blotting of liver extracts. Carboxyl-terminal, Stat5a-, or Stat5b-specific antisera were used to allow the detection of amino-terminal truncated proteins. As illustrated in Figure 1F, Stat5a or Stat5a/b mutant mice lacked detectable Stat5a protein, and Stat5b or Stat5a/b mutant mice lacked detectable Stat5b. Moreover, the disruption of one *Stat5* gene did not affect the expression of the remaining gene. Although not evident in the data presented, we have detected truncated Stat5a and Stat5b proteins on overexposed blots that are estimated to occur at 1% or less of the normal levels.

Since the *Stat3* gene is approximately 5 kb from the *Stat5a* gene, oriented in a tail-to-tail configuration (unpublished data), we examined the effects of the gene disruptions on Stat3 levels. As illustrated in Figure 1F, Stat5a mutant mice and the Stat5a/b mutant mice had somewhat lower levels of Stat3. The complete disruption of Stat3 results in an early embryonic lethality (Takeda et al., 1997), indicating that the decrease is unlikely to be significant. Lastly, there was a small increase in the Stat1 levels that was comparable to those previously observed in the Stat5b-deficient mice (Udy et al., 1997).

Effects of Stat5 Gene Disruptions on Hematopoiesis

The activation of the Stat5 proteins by Epo, Tpo, and other hematopoietic cytokines prompted us to analyze in some detail the hematopoietic functions in the mutant mice. There were no significant differences in several parameters, including the number of red cells, hemoglobin levels, or the hemocrits among the wild-type mice and the Stat5 mutant mice (data not shown). Similarly, there were no significant differences in the numbers of platelets between wild type mice and the various mutant mice. Lastly, in the Stat5a/b mutant mice, but not in Stat5a or Stat5b mutant mice, there was a decrease in the white cell count that was associated with a decrease in the percentage of lymphocytes. Conversely, there was an increased percentage of neutrophiles. The basis of the lymphocytopenia is considered below. Thus, in terms of peripheral myelopoiesis, the various Stat5 mutant mice have normal levels of erythrocytes, platelets, neutrophiles, and monocytes.

We also examined bone marrow hematopoietic progenitors in colony assays (Table 1). Consistent with the above, there was no detectable alteration in the number of erythroid colony-forming cells (CFU-E) in any of the mutant mice. However, there was a detectable reduction in the number of colonies induced in response to IL-3 (57% of wild type), IL-5 (25% of wild type), or GM-CSF (47% of wild type), and the size of the colonies was detectably smaller. The response of bone marrow cells to G-CSF–induced colony formation was also reduced to a comparable extent. Since G-CSF primarily induces Stat3 activation (Tian et al., 1994; Tweardy et al., 1995), it is possible that the reduction in colonies is secondary to other effects. Lastly, there were no detectable alterations in the responses to colony-stimulating factor 1 (CSF-1) or stem cell factor (SCF), two cytokines that utilize tyrosine kinase receptors.

IL-7 also activates Stat5 and is critical for early lymphoid expansion. In none of the mutant mice was there a detectable decrease in thymocytes or peripheral B cells (data not shown). However, as indicated in Table 1, the number of IL-7–induced bone marrow colonies in Stat5a/b mutant mice was 27% of control and has varied in various experiments from this value to approximately 40% of control. However, the number of pre–B cells (B220$^+$, CD43$^-$,IgM$^-$) and B cells (B220$^+$,CD43$^-$,IgM$^+$), as assessed by FACS analysis, was reduced by 64% and 36%, respectively. Since the Stat5a/b mice have a profound deficiency in peripheral T cell proliferation (unpublished data), it is possible that the reductions may be secondary to these defects.

Disruption of Stat5a or Stat5b Differentially Affect Mammary Gland Development

Whole mammary gland mounts and histological sections are illustrated in Figure 2 for mature virgin mice of all mutant strains and for the lactating Stat5a and Stat5b mutant mice. As indicated below, female Stat5a/b mice are infertile and therefore could not be examined. As illustrated, the differentiation of ductal elements occurs in all the mutant mice comparable to wild-type mice. The major difference is the extent of development of terminal buds in all mutant mice. Following pregnancy,

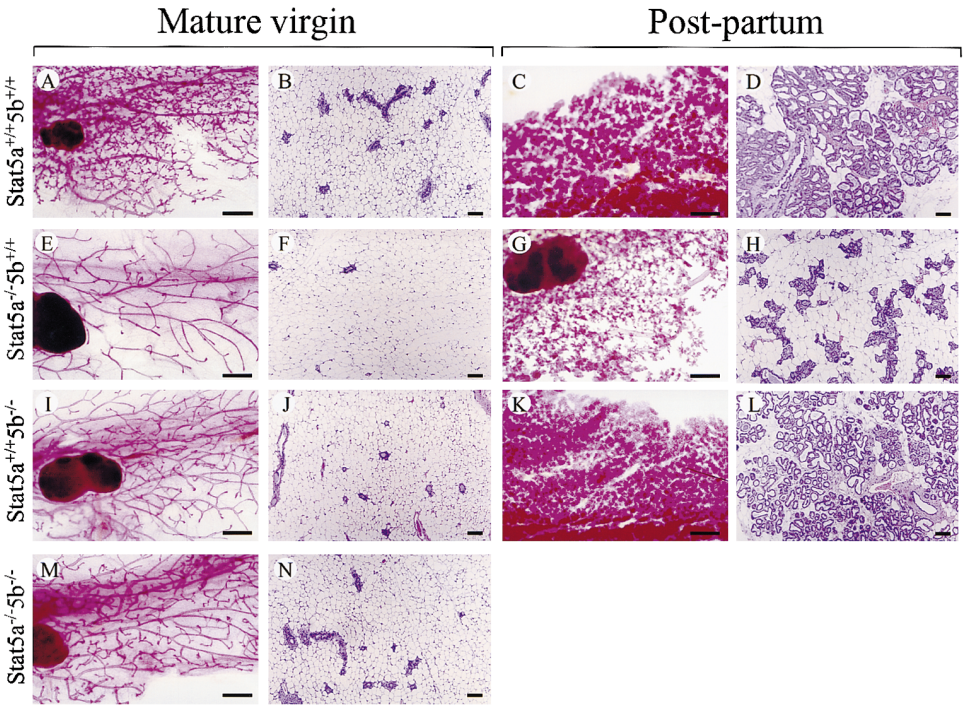

Figure 2. Histology of Mammary Glands from Stat5 Female Mutant Mice

(A, B, C, and D) Wild-type. (E, F, G, and H) Stat5a$^{-/-}$. (I, J, K, and L) Stat5b$^{-/-}$. (M and N) Stat5a$^{-/-}$5b$^{-/-}$. Mature virgin female mice are 12–14 weeks of age, and postpartum denotes 12 hr after delivery by 8- to 12-week-old female. (A, C, E, G, I, K, and M) Mammary gland whole mounts stained with carmine. Bar, 1 mm. (B, D, F, H, J, L, and N) 4 μm sections stained with hematoxylin-eosin. Bar, 100 μm.

in response to prolactin, the alveolar development of the mammary gland is greatly amplified in wild-type mice (Figures 2C and 2D). This expansion occurs relatively normally in Stat5b mice (Figures 2K and 2L) but is dramatically reduced in Stat5a mice (Figures 2G and 2H).

The development of the mammary gland is dependent upon the level of prolactin signaling, as indicated by the lack of lactation when a single prolactin receptor allele is disrupted (Ormandy et al., 1997). Thus, the differential effects of Stat5a and Stat5b deletion could be due to protein levels or extent of activation. Tyrosine phosphorylation of both Stat5a and Stat5b is detected in wild-type animals at 12 or 36 hr postpartum (Figure 3). Using an excess of anti-Stat antibodies, there was a reproducibly 3- to 5-fold lower level of tyrosine phoshorylated Stat5b, and the levels of Stat5b protein were approximately 2- to 3-fold lower. These levels were not altered in the mutant mice. These data suggest that the phenotypic differences are related to the higher level of expression of Stat5a rather than a isotype-specific function.

We also examined the levels of milk protein production (Figure 3B). The absence of either Stat5a or Stat5b resulted in a significant decrease in α-lactalbumin relative to wild-type mice, both at 12 and 36 hr of lactation, despite the differences in the gross mammary gland development. A comparable pattern was seen in the expression of WAP (whey acidic protein), although, with the mammary gland development that occurs in the Stat5b mutants, the levels of WAP increased to normal levels. In contrast, there was a much less dramatic effect of mutation of Stat5a or Stat5b on β-casein. The results demonstrate that both Stat5a and Stat5b contribute to α-lactalbumin and WAP expression, redundantly. However, neither plays a major role in β-casein expression.

Stat5a/b Mutants Have Altered Ovarian Development and Are Infertile

As noted above, the Stat5a/b mutant female mice, but not the Stat5a or Stat5b mutants, were infertile. The basis for the infertility was readily evident from the histology of the ovaries (Figures 4A and 4B). In both wild-type and mutant mice, developing follicles were evident, including mature Graafian follicles. Examination of the oviducts of Stat5a/b mutant mice also indicated that ovulation can occur normally (data not shown). However, whereas ovaries from wild-type mice invariably had large corpora lutea, there were either few or no large corpora lutea evident in the Stat5a/b mutant mice.

To further relate Stat5 function to ovarian development, we identified the sites within the ovary where Stat5 activation was occurring by taking advantage of the observation that Stat5 induces the gene "cytokine-inducible SH2-containing protein" (*CIS*) (Yoshimura et al., 1996). By in situ hybridization (Figure 4C), the major site of expression is in the corpora lutea and not in developing or mature follicles. Consistent with a role for the Stat5 proteins, no *CIS* expression was detected in any structures in the ovaries of Stat5a/b mutant mice (Figure 4D). In the presence of prolactin, follicle granulosa cells develop into a functional corpus luteum. In the absence of sufficient prolactin, the granulosa cells differentiate to copora lutea atretica. Functional corpora

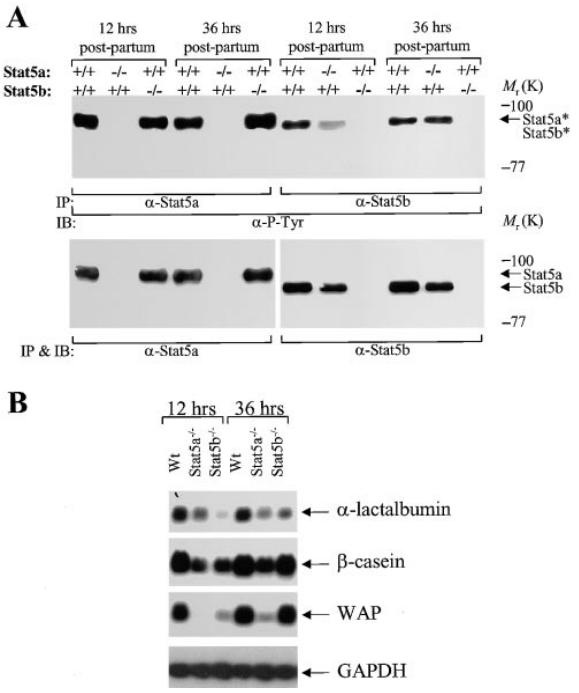

Figure 3. Mammary Gland Expression of Stat5a, Stat5b, and Milk Protein Genes in Stat5 Mutant Mice

(A) Western blot analysis of mammary gland extracts from wild-type, Stat5a$^{-/-}$, and Stat5b$^{-/-}$ mice at 12 and 36 hr postpartum immunoprecipitated (IP) with antisera specific for Stat5a and Stat5b, and immunoblotted (IB) with anti-phosphotyrosine sera (top panel). The blot was stripped and reprobed with the Stat5a- and Stat5b-specific antisera (bottom panel).

(B) Northern blot analysis of total RNA (15 μg/lane) from wild-type, Stat5a$^{-/-}$, and Stat5b$^{-/-}$ mice at 12 and 36 hr postpartum. Milk protein gene expression were assessed by successive hybridization with oligonucleotide probes for α-lactalbumin, β-casein, and whey acidic protein (WAP). Reprobing with GAPDH cDNA probe served as control for loading.

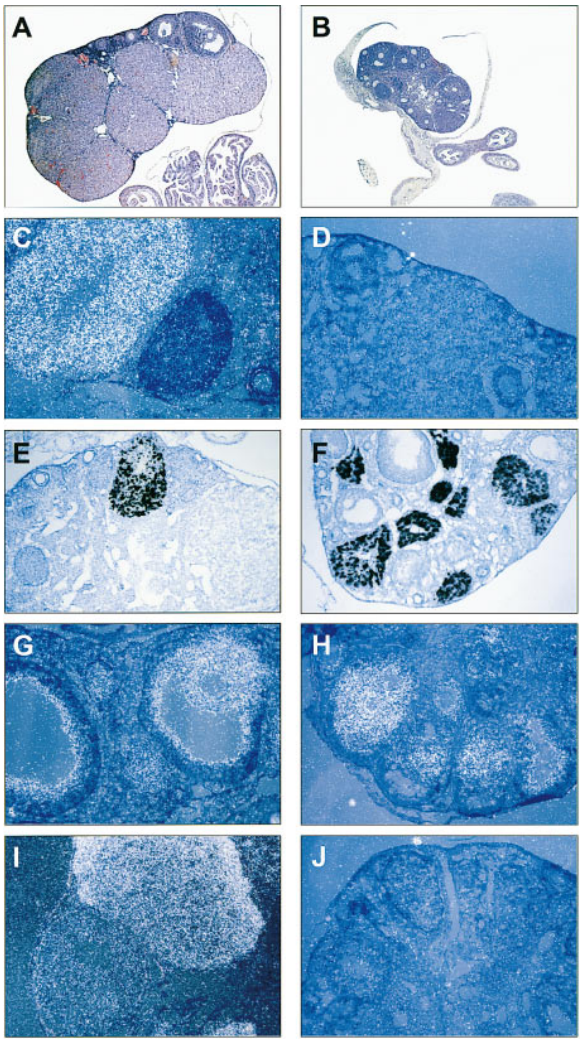

Figure 4. Histology and In Situ Hybridization of Wild-Type and Stat5a$^{-/-}$5b$^{-/-}$ Ovaries

(A, C, and E) Wild-type and (B, D, and F) Stat5a$^{-/-}$5b$^{-/-}$ ovaries from 8-week-old female mice. (A and B) Hematoxylin-eosin–stained 6 μm sections. (C and D) Dark-field image of in situ hybridization with Cis cRNA probe (6 μm sections). (E and F) Bright-field image of in situ hybridization with 20α-hydroxysteroid dehydrogenase cRNA probe (6 μm sections). (G and H) Dark-field image of in situ hybridization with Cyclin D2 cRNA probe. (I and J) Dark-field image of in situ hybridization with p27 cRNA probe.

lutea synthesize progesterone, while copora lutea atretica express the enzyme 20-α-hydroxysteriod dehydrogenase (20αSDH), which converts progesterone to an inactive derivative. Suppression of 20αSDH expression has been proposed to be due to prolactin signaling (Zhong et al., 1997). By in situ hybridization (Figure 4E), 20αSDH was not expressed in the corpora lutea or follicles but rather in the copora lutea atretica. In the Stat5a/b mutant mice, the 20αSDH-expressing structures were much more prevalent. The results suggest that Stat5 mediates a prolactin-induced signal that is critical for the differentiation of functional copora lutea and/or suppression of differentiation to copora lutea atretica. Alternatively, the Stat5 proteins may directly suppress 20αSDH expression, and the attendant changes in progesterone concentrations may affect differentiation.

Recently, cyclin D2 and p27 have been shown to be required for ovarian function by gene disruptions (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; Sicinski et al., 1996). To assess the relationship of these gene products to Stat5, we used in situ hybridization to examine cyclin D2 and p27 expression in wild-type and mutant mice (Figures 4G and 4H). Cyclin D2 expression was detected only in the granulosa cells of developing follicles, and this expression was not altered in Stat5a/b mutant mice. In contrast, p27 is highly expressed in corpora lutea of wild-type mice. Moreover, p27 expression was not detectable in any of the structures of the ovaries of Stat5a/b-deficient mice, suggesting a role for the Stat5 proteins in p27 expression but not cyclin D2.

Stat5a and Stat5b Are Essential for Growth Hormone Functions

The potential roles of the Stat5 proteins in mediating growth hormone effects were readily evident from the size of the mice (Figure 5). The most dramatic phenotype

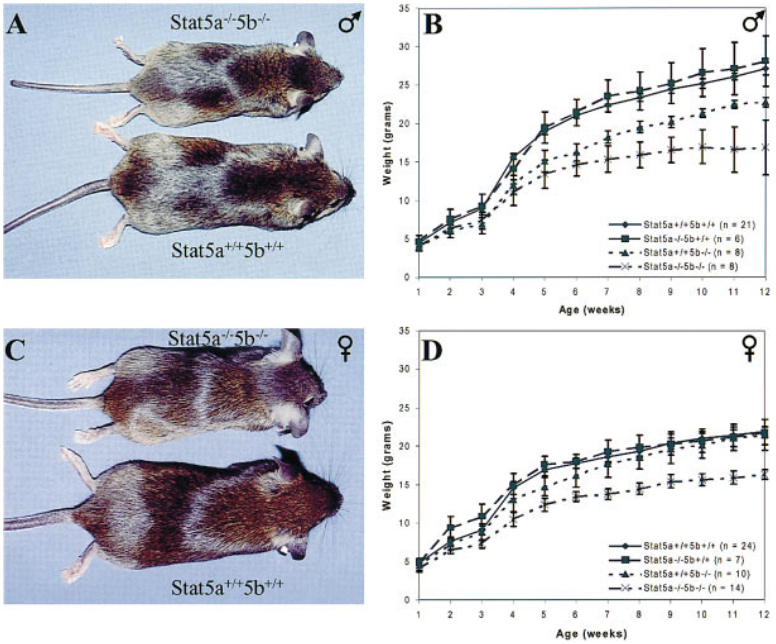

Figure 5. Body Growth Rates of Stat5 Mutant Mice

(A) Representative male littermates; wild-type (bottom) and Stat5a$^{-/-}$5b$^{-/-}$ (top) at 14 weeks of age.

(B) Growth curves of wild-type, Stat5a$^{-/-}$, Stat5b$^{-/-}$, and Stat5a$^{-/-}$5b$^{-/-}$ male mice from 1 through 12 weeks of age.

(C) Representative female littermates; wild-type (bottom) and Stat5a$^{-/-}$5b$^{-/-}$ (top) at 8 weeks of age.

(D) Growth curves of wild-type, Stat5a$^{-/-}$, Stat5b$^{-/-}$, and Stat5a$^{-/-}$5b$^{-/-}$ female mice from 1 through 12 weeks of age.

was observed in Stat5a/b mutant mice. At 12 weeks of age, the male and female mice weighed 30%–40% and 20%–30% less than their wild-type littermates. No effect was seen in the Stat5a mutant mice, while the Stat5b male mice were consistently 20%–30% smaller than their wild-type littermates. The Stat5a/b mutant phenotypes are quite similar to those observed in growth hormone (Donahue and Beamer, 1993) or growth hormone receptor–deficient mice (Zhou et al., 1997). The growth hormone receptor–deficient mice also display a decrease in fat deposition early in life (unpublished data). Consistent with this, Stat5a and Stat5b mutant mice had somewhat reduced epidemal fat pads, while the fat pads of Stat5a/b mutant mice were approximately one-fifth that of wild type (data not shown).

Some of the effects of growth hormone are related to induction of insulin-like growth factor 1 (IGF-1). As a consequence, growth hormone receptor–deficient mice have reduced serum IGF-1 (Zhou et al., 1997). IGF-1 levels were significantly reduced in Stat5a/b mutant male relative to wild-type (173 ± 23 vs. 340 ± 6) and female (127 ± 53 vs. 284 ± 40) mice. The levels of serum IGF-1 were reduced in male Stat5b mutant mice (175 ± 18) but not in the females, while the levels were normal in both male and female Stat5a mutant mice. Growth hormone has also been implicated in the regulation of a number of liver genes that are expressed in a sexually dimorphic pattern (Udy et al., 1997), and Stat5b has been implicated in this expression. The pattern of the major urinary protein (MUP) is illustrated in Figure 6A. Female wild-type mice express approximately 3- to 5-fold lower levels of MUP than males. In males, the deletion of Stat5a alone or Stat5b alone resulted in no changes in MUP levels. However, the deletion of both genes resulted in a dramatic reduction in MUP levels in males. In females, the deletion of Stat5a alone had no effect on MUP levels, while the deletion of Stat5b resulted in a significant decrease in MUP expression. However, the deletion of both Stat5a and Stat5b resulted in undecteable levels of MUP proteins in females.

We also examined the expression of male predominant and male suppressed genes (Figure 6). The P450 gene *CYP2D9* is expressed at higher levels in male mice relative to females (please note that the *CYP2D9* gene product is the protein that migrates slightly below the upper protein). This expression is lost in the Stat5b and the Stat5a/b mutant male mice and, to a lesser extent, in the Stat5a mutant male mice. Conversely, the P450 gene *CYP2A4* is normally expressed at higher levels in females relative to males. As illustrated in Figure 6B, the Stat5b mutant male mice have levels of CYP2A4 that are similar to the levels seen in female wild-type and Stat5a/b mutant mice. A similar increase in CYP2A4

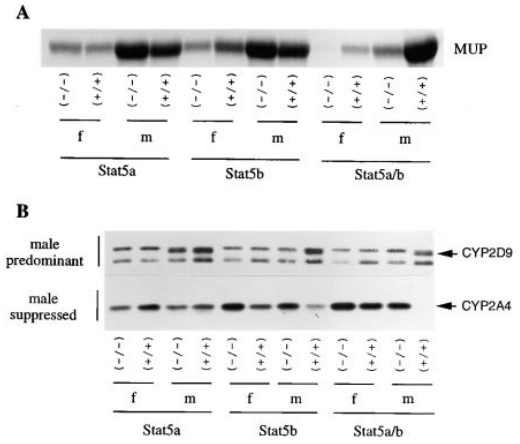

Figure 6. Regulation of Hepatic Gene Expression by Stat5b

(A) Urine (1 μl) from Stat5a, Stat5b, and Stat5a/b (+/+) and (−/−) male and female mice resolved by SDS polyacrylamide gel electrophoresis and stained with Coomassie blue.

(B) Immunoblot analysis of liver microsomes (10 μg) from Stat5a, Stat5b, and Stat5a/b (+/+) and (−/−) male and female mice with antibody to female predominant (male suppressed) testosterone 15α-hydroxylase (CYP2A4) or to male predominant testosterone 16α-hydroxylase (CYP2D9).

expression was not seen in the Stat5a mutant males. Similar results were obtained with several additional male suppressed P450 genes, including *CYP3A* and *CYP4A* (data not shown).

Discussion

The results demonstrate that Stat5a and Stat5b are required, either individually or redundantly, in virtually all the physiological responses associated with either growth hormone or prolactin. Conversely, it is remarkable that the Stat5 transcription factors play either no role, or redundant roles, in the response of a number of related cytokines that activate their transcriptional function.

Perhaps the most striking observation is the lack of any effect on Epo functions. There were no detectable alterations in red cell production in vivo nor were there any detectable effects on Epo-induced colonies from bone marrow cells. This is surprising, since numerous studies have implicated Stat5 in proliferation and/or differentiation (Damen et al., 1995; Gobert et al., 1995; Mui et al., 1996; Ogawa et al., 1996; Iwatsuki et al., 1997; Wakao et al., 1997; Woldman et al., 1997). However, the effects were always partial, and therefore it may be necessary to study erythroid differentiation in the mutant mice in additional ways to detect subtle effects.

The receptors for Epo and Tpo, as well as growth hormone and prolactin, are structurally similar and may have been derived from a primordial receptor. Because of this, it is more striking that, while growth hormone and prolactin require the Stat5 proteins, Epo and Tpo do not. It is possible that through evolution the receptors maintained, or acquired, the tyrosines critical for the recruitment of the Stat5 proteins, although functionally, the requirement for the Stat5 proteins was only retained, or acquired, in the responses to growth hormone or prolactin. Alternatively, the responses to Epo or Tpo could have evolved backup, redundant signaling pathways to the Stat5 proteins.

There were detectable effects on some cytokine colony-forming responses of bone marrow cells from Stat5a/b mutant mice, including IL-3 and GM-CSF, and thus the Stat5 proteins may contribute redundantly to these responses. A reduction in these activities would not be anticipated to have a physiological consequence, since mice lacking the ability to respond to IL-3, GM-CSF, as well as IL-5, have no hematopoietic phenotypes (Nishinakamura et al., 1996). The possibility that the effects are not directly due to absence of Stat5 proteins is suggested by decreased response to G-CSF, which predominantly activates Stat3. The possiblity exists that immune defects described below indirectly affect the levels of bone marrow progenitor cells.

The Stat5 proteins are essential for the development of functional corpora lutea in the ovary. This is consistent with the critical role that prolactin has in ovarian function based on the female infertility of both prolactin-deficient mice (Horseman et al., 1997) and prolactin receptor–deficient mice (Ormandy et al., 1997). Female infertility was not observed in the Stat5a or Stat5b mutant mice, demonstrating the functional redundancy of the Stat5 proteins. The critical targets of this signaling pathway are not known. A role in cyclin D2 regulation is unlikely, since D2 is not expressed where Stat5 activation occurs and its expression requires a follicle stimulating hormone (FSH)–dependent, cAMP pathway (Sicinski et al., 1996). In contrast, p27 is expressed at high levels in the corpora lutea, and the disruption of the *p27* gene results in an ovarian defect that is similar to that observed in the Stat5a/b-deficient mice (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). It is tempting to speculate that the Stat5 proteins regulate p27 expression and that this expression is critical for inducing the differentiation of granulosa cells along a pathway for the formation of functional corpora lutea.

Previous studies (Zhong et al., 1997) suggested that prolactin is required for the suppression of *20αSDH* gene expression. As demonstated here, 20αSDH is not expressed in the developing follicles or in the functional corpora lutea but rather in corpora lutea atretica. In the Stat5a/b mutant mice, there are many more 20αSDH expressing corpora lutea atretica. Thus, the Stat5 proteins are either critical for inducing the formation of functional corpus lutea and that, associated with this, is the lack of induction of 20αSDH. Alternatively, activation of the Stat5 proteins in the functional corpus lutea may be responsible for the suppression of 20αSDH expression.

The basis for the infertility of Stat5a/b mutant mice can be attributed to the absence of sufficient progesterone to support implantation and subsequent placental development and maintanence. The importance of progesterone in ovarian and uterine functions is evident from the phenotype of progesterone receptor–deficienct mice (Lydon et al., 1995). The primary site of production of progesterone during pregnancy is the corpora lutea of the ovary. The lack of development of corpora lutea would therefore be expected to have a significant effect on the levels of progesterone. In addition, the accumulation of corpora lutea altricans, which express high levels of 20αSDH, would further contribute to decreasing the levels of progesterone.

The phenotype of growth hormone receptor–deficient mice has recently been described (Zhou et al., 1997). Some of the phenotypes are observed in Stat5b mutant mice, while all the phenotypes are evident in the Stat5a/b mutant mice. Interestingly, the principle differences were in females with only a Stat5b mutation, which showed normal IGF-1 levels and growth. These differences may also be due to the differences of the Stat5a and Stat5b levels. Specifically, Stat5b is much more highly expressed in the liver than Stat5a (Ripperger et al., 1995). In contrast Stat5a and Stat5b levels are comparable in ovary (data not shown). In this context, the observation that the corpus luteum produces IGF-1 (Parmer et al., 1991) helps to explain the sex differences in the phenotype. Thus, in males, the primary source of IGF-1 is the liver, while in females both liver and ovaries can contribute. The ovarian contribution is only eliminated by the absence of both Stat5a and Stat5b.

The data suggest that most of the physiological responses to growth hormone involve receptor activation of Stat5 proteins by Jak2. These observations are not consistent with recent experiments concerned with growth hormone–induced tyrosine phosphorylation of the epidermal growth factor (EGF) receptor (Yamauchi et al., 1997). The authors concluded from their studies

that growth hormone–induced tyrosine phosphorylation of the EGF receptor and its ability to activate MAP kinases were critical to growth hormone function. From the phenotype of the Stat5a/b-deficient mice, it is not clear what growth hormone functions might be dependent upon other signaling pathways.

The results described here have focused on the phenotypes observed in the mutant mice that are directly attributable to deficiencies in growth hormone and prolactin function. In addition, however, the Stat5a/b mutant mice, but not the individual mutants, have a profound defect affecting the T cell lineage (unpublished data). Specifically, peripheral T cells are unable to proliferate in response to T cell receptor engagement and IL-2, although IL-2 receptor expression is unaltered and the T cells are able to produce cytokines. This aspect of the defects of Stat5a/b mutant mice strikingly resembles that seen in IL-2 receptor β chain–deficient mice (Suzuki et al., 1995). In summary, the phenotypes of the Stat5a/b mice illustrate remarkably specific functions for the Stat5 proteins in mediating virtually all growth hormone and prolactin functions, but, in addition, one can hypothesize that during the evolution of T cell function the Stat5 genes were incorporated into T cell regulation.

Experimental Procedures

Stat5a and Stat5b Gene Disruptions

The *Stat5a* and *Stat5b* genes were isolated from a 129/SvE mouse genomic library in λEMBL3. For the Stat5a targeting vector, a 9.3 kb EcoRI/SalI fragment was subcloned, followed by replacement of a 1.0 kb XhoI/HindIII fragment containing the two first *Stat5a* coding exons with a hygromycin resistance cassette (van Deursen et al., 1991). For the *Stat5b* targeting vector, a 1.6 kb NheI/ApaI fragment and a 6.3 kb XhoI/EcoRI fragment were subcloned 5′ and 3′, respectively, of a neomycin resistance cassette from pMC1neo-polyA (Stratagene), thereby leaving out the first *Stat5b* coding exon. A herpes simplex virus thymidine kinase (HSV-tk) cassette mediating negative selection was inserted in the 5′- and 3′-end of the Stat5b-neo and Stat5a-hygro construct, respectively.

E14 (129/Ola mouse strain) or RW4 (129/SvJ mouse strain [Genome Systems]) embryonic stem (ES) cells were cultured as previously described (Parganas et al., 1998). To generate the Stat5a$^{+/-}$5b$^{+/-}$ ES clones, Stat5a$^{+/-}$ hygro^{R} ES cells were targeted a second time now with the Stat5b-neo construct and double-resistant clones selected. Conditions for blastocyst injection were essentially as described (van Deursen et al., 1993). Ten, nine, and fifteen independent ES clones were injected for Stat5a, Stat5b, and Stat5a/b, respectively, of which two, three, and four clones, respectively, gave germline transmission. In three of the Stat5a$^{+/-}$5b$^{+/-}$ ES clones, targeting had occurred on the same chromosome as revealed by the F1 offspring genotypes. Tail DNA was prepared as described (Hogan et al., 1994). Genotyping of mice was performed by Southern blot analysis or by PCR. For PCR, the primers consisted of Stat5a: F9 primer (5′-AAGGGACAGGAAGAGAGAAGG-3′), R1 primer (5′-CCCA TACAACACTTGCATCT-3′); TK: TKp primer (5′-GCAAAACCACAC TGCTCGAC-3′); Stat5b: R8 primer (5′-GGAGATCTGCTGGCTGA AAG-3′), F11 primer (5′-TCAAACACACCTCAATTAGTC-3′).

To detect Stat5a homologous targeting by Southern blotting, a HindIII digest was probed with a 500 bp KpnI fragment giving a 6.6 kb wild-type and a 7.8 kb mutant allele. To detect Stat5b homologous targeting, a XbaI digest was probed with a 900 bp EcoRI/KpnI fragment giving a 9.5 kb wild-type and a 25 kb mutant allele.

Bone Marrow Colony Assays

Bone marrow cells were prepared from femurs in α-MEM medium (GIBCO-BRL) containing 2% fetal bovine serum (FBS) (StemCell Technologies) and counted in the presence of 3% acetic acid to lyse the erythrocytes. Diluted cell suspensions and recombinant cytokines were mixed with MethoCult M3230 (StemCell Technologies) except for the pre–B cell assay (see below) giving a final concentration of 0.9% methylcellulose. Assays were plated in 35 mm culture dishes in duplicate and cultured at 37°C. CFU-E assays contained 5×10^4 cells/dish and 0.2 U/ml recombinant human erythropoietin, rhEpo (Amgen). Benzidine-positive CFU-E colonies were scored at day 3. BFU-E assays contained 2×10^5 cells/dish and 3 U/ml rhEpo and 10 ng/ml recombinant murine IL-3, rmIL-3 (R&D Systems). Benzidine-positive BFU-E colonies were scored at day 8. CFU-Mix assays contained 1.5×10^4 cells/dish and 10 ng/ml rmIL-3 or 2×10^5 cells/dish and 50 ng/ml rmSCF (R&D Systems). Colonies were scored at day 12. CFU-Meg assays contained 4×10^5 cells/dish and 50 ng/ml recombinant human thrombopoietin, rhTpo (Genzyme). Colonies were scored at day 8. CFU-GM and CFU-M assays contained 1.5×10^4 cells/dish and 10 ng/ml rmGM-CSF or 10 ng/ml rmCSF-1 (R&D Systems). Colonies were scored at day 12. CFU-G assays contained 5×10^4 cells/dish and 10 ng/ml rhG-CSF (Amgen) and were scored at day 12. CFU-Eos assays contained 2×10^5 cells/dish and 20 ng/ml rmIL-5 (R&D Systems), and colonies were scored at day 18. Pre–B cell assays contained 5×10^4 cells/dish and were cultured in MethoCult M3630 (StemCell Technologies) including 10 ng/ml rhIL-7 and colonies scored at day 8.

Histology and In Situ Hybridization

Mammary glands for whole mounts were spread on glass slides and fixed 4 hr in Carnoy's fixative. The tissue slides were sequentially rehydrated 15 min each in a graded ethanol (70%, 50%, 30%, 10%, and H_2O) and stained overnight (0.2% w/v carmine [Sigma]/0.5% w/v aluminum potassium sulfate [Sigma]). The slides were dehydrated 15 min each in 70%, 95%, and 100% ethanol (2 times) followed by clearing in Hemo-De (Fisher). For sectioning, tissues were fixed in 10% phosphate-buffered formalin (Fisher), paraffin embedded, sectioned at 4 μm (mammary glands) or 6 μm (ovaries), and stained in hematoxylin and eosin.

For in situ hybridization, ovaries were fixed by perfusion with 4% paraformaldehyde (Sigma), and 6 μm sections were prepared from frozen tissue samples. In situ hybridization was carried out essentially as described (Angerer and Angerer, 1992) using [γ-^{33}P]UTP-labeled antisense RNA probes transcribed from plasmids containing murine cDNA fragments of *Cis* (216 bp), 20α-hydroxysteroid dehydrogenase (670 bp), Cyclin D2 (1.2 Kbp), and p27 (591 bp). Tissue sections were counterstained using toluidine blue (Sigma).

Immunoprecipitation and Western Blot Analysis

Total cell extracts were prepared from tissue homogenized in 10 mM Tris-HCl (pH 7.6)/5 mM EDTA/50 mM NaCl/30 mM sodium pyrophosphate/50 mM sodium fluoride/1 mM sodium orthovanadate/10% glycerol with Complete protease inhibitor cocktail (Boehringer Mannheim). The methods used to detect Stat proteins have been described (Quelle et al., 1995; Wang et al., 1996). For analysis of major urinary proteins (MUPs), 1 μl of mouse urine was resolved by 12% SDS-PAGE followed by Coomassie blue staining (Norstedt and Palmiter, 1984). For the P450 analysis, liver microsomes were prepared and 10 μg analyzed on immunoblots using rabbit antibodies to either female predominant testosterone 15α-hydroxylase (CYP2A4) (Squires and Negishi, 1988) or male predominant testosterone 16-hydroxylase (CYP2D9) generously provided by Dr. M. Negishi (NIEHS, Research Triangle Park, NC), followed by HRP-conjugated anti-rabbit IgG and ECL.

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